METHOD OF PLASTID TRANSFORMATION IN *ASTERACEAE*, VECTOR FOR USE THEREIN AND PLANTS THUS OBTAINED

Field of the invention

The invention relates to methods of genetically transforming plant plastids, and more specifically to genetically transforming the plastid genomes of *Asteraceae* plant species. The invention further relates to vectors for use in the transformation of plastid genomes and to transplastomic plants thus obtained and their progeny.

Background of the invention

Plastids are self-replicating organelles containing their own DNA in a single circular chromosome, called their genome. Plastids are found in all plant cells. They are inherited maternally in most plants just like mitochondria in animals and plants. This is also called cytoplasmic inheritance since these organelles are present in the cytosol of the ova.

Plant plastids (e.g. chloroplasts, amyloplasts, elaioplasts, etioplasts, chromoplasts, leucoplasts and proplastids) are the organelles in which major biochemical processes (i.e. photosynthesis) take place. In general, plant cells contain between 100-10,000 copies of the small 120-160 kb circular plastid genome. Since each molecule has one inverted repeat it is theoretically possible to obtain plant cells with 20,000 copies of (a) gene(s) of interest, after plastid transformation.

The genetic transformation of the plastid genome (plastome) has major advantages over nuclear transformation. Firstly, because in most plant species, plastids are maternally inherited, out-crossing of transgenes to weeds or other crops is minimized. Thus, this form of genetic

engineering of plants lowers the risk of dissemination of the transgene in the environment through pollen dispersal. Furthermore, the plastid genome is highly polyploid, enabling the introduction of many copies per cell which can lead to high accumulation levels of the desired protein(s). The fact that plastids are able to form disulfide bonds and to fold proteins, makes this technique in theory ready for the production of biopharmaceuticals in plants.

The principle of plastid transformation is insertion of sequences through homologous recombination. Plastid transformation vectors use two targeting DNA segments that flank the gene or genes of interest. By means of homologous recombination these segments can insert the foreign gene or genes at a precise, predetermined position in the plastid genome. Position effects and gene silencing, major problems in nuclear transformation experiments, have not as yet been observed in plastid transformation events.

However, successful chloroplast transformation of crop plants is described thus far only for Solanaceous crops like potato, tomato, tobacco (US-5,451,513; Svab et al. (1990), Proc. Natl. Acad. Sci. USA 87:8526-8530) and *Brassicaceae*, like *Arabidopsis thaliana* (US-6,376,744). It is not obvious that the techniques used for these species can be readily used for other species such as *Asteraceae*, in particular lettuce.

It is therefore the object of the invention to provide an alternative plastid transformation method that is in particular useful for transforming *Asteraceae* plant species, such as lettuce (*Lactuca sativa*). Lettuce is an agronomical important crop and a useful transformation method therefore is thus highly desirable.

Summary of the invention

The invention thus provides a method for the transformation of plastid genomes of plant species, in particular *Asteraceae* plant species, comprising the steps of:

a) providing a transformation vector;

b) subjecting a plant material, which comprises plastids, to a transformation treatment in order to allow the plastids to receive the transformation vector;

c) placing the thus treated plant material for a period of time into contact with a culture medium without selection agent;

d) subsequently placing the plant material into contact with a culture medium comprising a selection agent; and

e) refreshing the culture medium comprising a selection agent to allow plant material comprising plastids that have acquired the DNA of interest to grow into transformants, in particular transplastomic plants or plant parts (i.e. plants or plant parts carrying one or more transgenes in their plastids).

The transformation vector may comprise:

- an expression cassette which comprises optionally a promoter active in the plastids of the plant species to be transformed, a DNA insertion site for receiving the transforming DNA of interest, optionally one or more selection markers conferring a selectable phenotype on cells having plastids that are transformed with the expression cassette, and optionally a DNA sequence encoding a transcription termination region active in the plastids of the plant species to be transformed,

- optionally a set of DNA targeting segments located on either side of the expression cassette that allow double

homologous recombination of the expression cassette with the plastid genome of interest, and

- a DNA sequence of interest inserted into the insertion site of the expression cassette.

Preferably the vector comprises a promoter, a set of targeting segments and one or more selection markers. However, these elements may also be provided in another way. For example, the DNA of interest can be inserted at such a position in the plastome that it can use an already present promoter, such as in an operon. If no targeting segments are present the DNA of interest can integrate at a random position. The DNA of interest is preferably integrated in the plastid genome but can also exist outside the plastome.

The DNA of interest can be either stably integrated or transiently expressed.

It is surprising that when using the method of the invention no escapes are found in the transformation of plastids of lettuce. The results of plastid transformations thus far, mention the occurrence of escapes (due to nuclear or spontaneous mutants; Kofer et al. (1998) In Vitro Cell. Dev. Biol. Plant 34:303-309).

It was surprisingly found that not immediately starting the selection process but keeping the treated plant material in or on a culture medium for a few days highly improved the efficiency of transformation. In addition, the selection procedure should not be started too late in the culture process. Preferably, selection is started after a maximum of 2-5 days. The moment to start the selection process depends on the transformation method. Another important aspect of the invention is to keep the transformed cells into close contact with the selective agent for a period of time, preferably until regeneration. In addition, it is preferred to retain the concentration of the selective

agent at an efficient level, such as 500 mg/l spectinomycin dihydrochloride. This is preferably achieved by using a liquid medium containing the selective agent.

Detailed description of the invention

The invention provides methods and vectors for efficient and stable transformation of plastids of an *Asteraceae* plant species, in particular chloroplasts of a lettuce plant, and the plants thus obtained.

Other plastids that can be transformed by the method of the invention are selected from the group consisting of amyloplasts, elaioplasts, etioplasts, chromoplasts, leucoplasts and proplastids.

The vector that is used in the method of the invention has a vector backbone and in addition a DNA construct that optionally comprises one or more sets of targeting DNA segments that are homologous to a sequence in the plastid genome, optionally a promoter sequence, optionally a DNA sequence encoding the transforming gene inserted in an insertion site, optionally a terminator sequence, and optionally at least one DNA sequence encoding a selectable marker.

Preferably, the vector comprises the targeting DNA segments, the DNA sequence encoding the transforming gene, a promoter and a selectable marker.

The promoter is any promoter that is active in the plastids of the plant species to be transformed and for lettuce for example selected from the group of (lettuce or other plant species) chloroplast specific ribosomal RNA operon promoter rrn (16S rRNA), psbA, rbcL, trnV, or rps16. However, additional promoter regions, to enhance transcription, translation or both processes, can also be used for obtaining expression of the selectable marker and

gene of interest in lettuce plastids. Also, bacterial promoters can be used for expressing genes in the plastids.

The terminator is any terminator that is active in the plant species to be transformed and for lettuce for example selected from the group consisting of the *psb* A termination sequence, rrn, rbcL, trnV, or rps16. These and other terminators may be specific for lettuce or other plant species. A terminator sequence need not always be present in bicistronic constructs, being two open reading frames behind one promoter. Additional UTR (untranslated region) sequences, fused to coding sequences of desired gene(s), can be used as leader and/or trailer, to minimize unwanted recombination.

The selection marker is for example selected from the group consisting of spectinomycin, streptomycin, kanamycin, hygromycin and chloramphenicol, or to plant herbicides like glyphosate or bialaphos. Of these markers the aadA gene is preferred because it is a non-lethal marker.

Alternatively a visual marker can be used, such as gfp (green fluorescence protein). In that case the selective agent is not a compound or composition but the means that is used to visualize the visual marker, such as the source of blue light that leads to fluorescence of the gfp.

When only such visual marker is used for selecting the transformants, steps d) and e) of the method can be performed without selective agent. The selection is then made visually by illuminating the putative transformants with an appropriate source of light and selecting the transformants that show fluorescence.

The DNA segments that allow double homologous recombination of the DNA of interest with the plastid genome of interest have a DNA sequence that is homologous to a part of the plastid genome. The segments are selected such that integration of the transforming gene takes place in a desired

position in the genome. For lettuce, for instance, the set of DNA segments is selected from the *trn*I(*ori*A)/*trn*A region and the 16S/trnV/ORF70B region of the lettuce chloroplast genome. Preferably, the set of DNA segments is selected from LCV1 A-B and LCV1 C-D, and LCV2 A-B and LCV2 C-D as disclosed in the Examples. The advantage of these segments is that they were found to be particularly useful for lettuce.

The method of the invention can be used for the preparation of plants that can express any gene of interest. The inventive technology can be used for the transformation of plastids from any plant, but in particular for plants of the *Asteraceae* family, more in particular for lettuce. The invention can thus be used for the production of polypeptides that can be isolated from the plant or of polypeptides that are useful for the plant itself. An example of production of products that can be isolated from the plant lies for example in the field of biopharmaceuticals, i.e. pharmaceuticals produced in living organisms such as plants. The production in plants has high potential because it can lead to lower production costs as compared to production in animals or in microorganisms using Bioreactors.

A promising new field in which this invention can be used is the production of edible vaccines, but other pharmaceuticals, either therapeutic or prophylactic, can be envisaged as well as (poly)peptides that can be used in other fields.

In addition to using the plant as a factory for the production of peptides or polypeptides, the product expressed can also be of agronomical importance. Examples are herbicide resistance, insect resistance, fungal resistance, bacterial resistance, stress tolerance for instance to cold, high salt or minerals, yield, starch accumulation, fatty acid accumulation, photosynthesis.

According to the invention, the transformation treatment is selected from the group consisting of electroporation, particle gun transformation, polyethylene glycol transformation and whiskers technology. Polyethylene glycol transformation and particle gun are very advantageous since a high number of cells can be transformed simultaneously and an efficient selection of the transformed plastids within the cells can take place.

The essence of the whiskers technology is the microscopic needle-like silicon-carbide "whiskers" which are approximately 0.6 microns in diameter and vary from 5-80 microns in length. The process begins with the provision of a "transformation cocktail" consisting of DNA, silicon carbide "whiskers", and the appropriate plant target tissue. This cocktail is then stirred or mixed or shaken in a robust fashion by a variety of means (such as a Vortex Machine, a Dental Amalgam Mixer, or a Commercial Paint Shaker). The resulting collisions between plant cells and "whiskers" are hypothesized to result in the creation of very small openings in the plant cell wall and membrane. As a consequence, DNA can move into the targeted plant cells, followed by integration of the transforming DNA into the plastome. Ultimately, transplastomic plant material can be recovered.

The period of time during which the treated plant material is placed into contact with a culture medium without selection agent depends on the transformation treatment. For polyethylene glycol transformation the period of time is 1 to 14 days, preferably 3 to 7 days, more preferably about 6 days. For particle gun transformation, the period of time during which the treated plant material is placed into contact with a culture medium without selection agent is 1 to 14 days, preferably 1-5 days, more preferably about 2 days. "Without selection agent" is intended to mean "without an

effective amount of the selection agent". During this period a low, i.e. ineffective amount of selective agent may be present.

The step of placing the treated plant material into contact with a culture medium without selection agent was found to be important for the transformation efficiency. In addition it is preferred for chloroplast transformation to keep the treated plant material in the dark during this step. This way no new and thus not transformed chloroplasts are produced thus leading to a higher efficiency.

The treated plant material is preferably kept into contact with a culture medium with the selection agent until regeneration of the plant or plant part from the transformed material.

The method of the invention is suitable for plant materials selected from plant tissue, separate cells, protoplasts, separate plastids.

It was surprisingly found that the transformation efficiency can be increased when the culture medium comprising the selection agent is a liquid medium. This way the cells to be transformed are in close contact with the selective agent. It was furthermore surprisingly found that no escapes were detected in the transformation experiments.

When the culture medium is refreshed after the selection procedure this may mean that fresh medium with selective agent is added (i.e. so that the selection medium is not diluted) or that the selection medium is changed for medium with selective agent.

The invention further relates to an expression vector for the transformation of plastid genomes of plant species, in particular *Asteraceae* plant species, which vector comprises:

- an expression cassette which comprises optionally a promoter active in the plastids of the plant species to be transformed, a DNA insertion site for receiving the transforming DNA of interest, optionally one or more selection markers conferring a selectable phenotype on cells having plastids that are transformed with the expression cassette, and optionally a DNA sequence encoding a transcription termination region active in the plastids of the plant species to be transformed,

- optionally a set of DNA targeting segments located on either side of the expression cassette that allow double homologous recombination of the expression cassette with the plastid genome of interest, and

- optionally a DNA sequence of interest inserted into the insertion site of the expression cassette.

In a preferred embodiment, the vector comprises the promoter, the one or more selection markers and the set of DNA targeting segments. Such vector comprises:

- an expression cassette which comprises a promoter active in the plastids of the plant species to be transformed, a DNA insertion site for receiving the transforming DNA of interest, one or more selection markers conferring a selectable phenotype on cells having plastids that are transformed with the expression cassette, and optionally a DNA sequence transcription termination region active in the plastids of the plant species to be transformed, and

- a set of DNA targeting segments located on either side of the expression cassette that allow double homologous recombination of the expression cassette with the plastid genome of interest.

The various elements of the vector are preferably as described above for the method. The invention relates both to

the vector in which no gene to be transformed is incorporated as well as to the vector comprising any transformable gene.

The vectors of the invention provide stable transformation of plastids of multicellular structures, such as plants of lettuce.

The invention further relates to plants carrying in their cells plastids that are transformed, in particular to plants carrying plastids transformed by means of the method of the invention. In addition, the invention relates to progeny of these plants in which at least part of the transformed plastids are still present.

The invention will be further illustrated in the Examples that follows. In these examples, as explant material, lettuce plant mesophyl protoplasts are used and via PEG transformation transplastomic protoplast-derived colonies and regeneration of plants were obtained. Alternatively, transplastomic callus was obtained using particle bombardment of excised cotyledons of lettuce. The DNA constructs comprise an expression cassette containing the transforming DNA which is targeted to a pre-determined location in the plastid genome and inserted into the plastid genome by homologous recombination. The targeting segments in the cassette comprise preferred sequences of the lettuce DNA chloroplast genome, i.e. the *trn*I(*ori*A)/*trn*A region or the 16S/trnV/ORF70B region of the lettuce chloroplast genome. The DNA used for transformation further contains a non-lethal selectable marker gene which confers a selectable phenotype on cells having the plastids with the transforming DNA, in this case spectinomycin. The non-lethal selectable coding sequence preferred, is the coding region of aadA from *E. coli*, which encodes aminoglycoside-3'-adenylyltransferase to confer spectinomycin and streptomycin resistance. Furthermore, the DNA expression cassette comprises at least one additional DNA

sequence, which is the DNA sequence of interest, such as a gene encoding a green fluorescent protein (gfp) (as a model system) or the influenza virus haemagglutinin gene (HA). The constructs furthermore are provided with a promoter and a terminator sequence functional in plant plastids.

In the Examples that follow reference is made to the following figures:

Figure 1. LCV1 lettuce chloroplast genome target sequence (not including backbone vector) (**SEQ ID NO:1**).

Figure 2. Map of LCV1 (7,545 bp).

Figure 3. LCV1 lettuce chloroplast genome target sequence (**SEQ ID NO:2**) aligned with tobacco chloroplast genome (GI Z00044) (**SEQ ID NO:3**). **SEQ ID NO:4** and **5** are the hypothetical proteins. **SEQ ID NO:41** is the ribosomal protein.

Figure 4. Cloning steps and primers (**SEQ ID NOS:6-9**) for construction of LCV1. TCG = tobacco chloroplast genome.

Figure 5. LCV2 lettuce chloroplast genome target sequence (not including backbone vector) (**SEQ ID NO:10**).

Figure 6. Map of LCV2 (6,182 bp).

Figure 7. LCV2 lettuce chloroplast genome target sequence (**SEQ ID NO:11**) aligned with tobacco chloroplast genome (GI Z00044) (**SEQ ID NO:12**).

Figure 8. Cloning steps and primers (**SEQ ID NOS:13-16**) for construction of LCV2. TCG= tobacco chloroplast genome.

Figure 9. Map of LCV1 MSK18 (9,682 bp).

Figure 10. Map of LCV2-MSK18 (8,329 bp).

Figure 11. Diploid Transplastomic lettuce pLCV2-LEC1 plants at stages of flowering (left upper panel), microspores (right upper panel) and seed set (right upper and lower panel)

Figure 12. Primer combinations (**SEQ ID NOS:17-20**) used in PCR analysis of transplastomic lettuce callus.

Figure 13. Molecular analysis of spectinomycin resistant lettuce calli.

Panel A: PCR products of the ATPase gene.

Lane 1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,
5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,
9 and 10 untransformed callus,
11 and 12 pLCV2-MSK18

Panel B: PCR products of the *Aad*A gene.

Lane 1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,
5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,
9 and 10 untransformed callus,
11 and 12 pLCV2-MSK18

Panel C: PCR products of the trnI junction.

Lane 1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,
5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,

9 untransformed callus

Panel D: PCR products of the trnA junction.

Lane 1. Marker,

2. TRSL5-01016 pLCV2-MSK18-1,

3. TRSL5-01016 pLCV2-MSK18-1

4. TRSL5-02002 pLCV2-MSK18-1-1,

5. TRSL5-02002 pLCV2-MSK18-1-2,

6. TRSL5-02002 pLCV2-MSK18-2-1,

7. TRSL5-02002 pLCV2-MSK18-2-1,

8. TRSL5-02002 pLCV2-MSK18-2-2,

9 untransformed callus

Figure 14. Sequence of left border (P1-P2) (**SEQ ID NO:21**) and right border (P3-P6) (**SEQ ID NO:22**) integration junction fragments amplified by PCR from transplastomic lettuce DNA. Sequence in lower case is lettuce chloroplast DNA external to the LCV2 vector target region. Upper panel: P1-P2 left border fragment consensus sequence; Lower panel: P3-P6 left border fragment consensus sequence.

Figure 15. Agarose gel electrophoresis of PCR products from reactions with primer pairs P1+P2, P3+P4 and P1+P4 and template DNA from spectinomycin resistant putative transplastomic callus sample B (TP) and non-transformed wild-type callus (WT).

Figure 16. PCR analysis on insert integration of pLCV2-MSK18 transformed calli. Lane 1: marker DNA, lanes 2-7: TRSL05-02002 pLCV2-MSK18-1-1, TRSL05-02002 pLCV2-MSK18-1-2, TRSL05-02002 pLCV2-MSK18-1-3, TRSL05-02002 pLCV2-MSK18-2-1, TRSL05-02002 pLCV2-MSK18-2-2, TRSL05-02001 pLCV2-MSK18-1-1, respectively; lane 8 and 9: control lettuce DNA, lane 10: plasmid DNA pLCV2-MSK18

Figure 17. PCR analysis of left and right border integration junction from callus, derived after particle bombardment transformation with plasmid pLCV2-MSK18.Panel A:

*trn*I junction (left integration junction). Panel B: *trn*A junction (right border insertion. Lane 1: lambda marker, lane 2: spectinomycin resistant callus pLCV2-MSK18, lane 3; control lettuce, lane 4: plasmid pLCV2-MSK18.

Figure 18. PCR analysis of pLCV2-LEC1 callus lines and controls.

A1: PCR products of the ATPase gene.

Lane 1; marker
2: pLCV2-LEC1 1.1
3: pLCV2-LEC1 2.1
4: pLCV2-LEC1 2.2
5: pLCV2-LEC1 3.1
6: pLCV2-LEC1 3.2
7: control non-treated lettuce callus
8: control untransformed callus

A2: PCR products of ATPase gene

Lane 1: marker
2: plasmid pLCV2-LEC1
3: water

B1: PCR products of the *Aad*A gene.

Lane 1; marker
2: pLCV2-LEC1 1.1
3: pLCV2-LEC1 2.1
4: pLCV2-LEC1 2.2
5: pLCV2-LEC1 3.1
6: pLCV2-LEC1 3.2

B2: PCR products of *Aad*A gene

Lane 1: marker
2: plasmid pLCV2-LEC1
3: water

C: PCR products of trnI junction (left border)

Lane 1; marker
2: pLCV2-LEC1 1.1

3: pLCV2-LEC1 2.1
4: pLCV2-LEC1 2.2
5: pLCV2-LEC1 3.1
6: pLCV2-LEC1 3.2
7: control non-treated lettuce DNA
8: plasmid pLCV2-LEC1

D: PCR products of *trn*A junction (right border)
Lane 1; marker
2: pLCV2-LEC1 1.1
3: pLCV2-LEC1 2.1
4: pLCV2-LEC1 2.2
5: pLCV2-LEC1 3.1
6: pLCV2-LEC1 3.2
7: control non-treated lettuce DNA
8: plasmid pLCV2-LEC1

E: PCR products of insert
Lane 1; marker
2: pLCV2-LEC1 1.1
3: pLCV2-LEC1 2.1
4: pLCV2-LEC1 2.2
5: pLCV2-LEC1 3.1
6: pLCV2-LEC1 3.2
7: control non-treated lettuce callus
8: control untransformed callus

Figure 19. PCR analysis on insert integration in 24 different transplastomic regenerants, originated from 1 transplastomic callus TRSL05-02002 pLCV2-MSK18 1-2 (Lanes A-L and M-X) and 2 control lettuce plants (control lettuce)

Figure 20. PCR analysis on insert integration in 7 different transplastomic regenerants, originated from 1 transplastomic callus number pLCV2-LEC1 2.2. Lane 1: marker, lanes 2-8: pLCV2-LEC1 2.2 regenerated plants, lane 9: plasmid DNA pLCV2-LEC1, lane 10: control lettuce DNA.

Figure 21. Lettuce expression cassette LEC1. LP*rrn* - lettuce specific RNA operon promoter; L3' *psb*A - lettuce specific *psb*A terminator sequence.

Figure 22. Schematic representation of the PCR and cloning strategy used for LEC1 construction together with primer sequences (**SEQ ID NOS:23-30**).

EXAMPLES

EXAMPLE 1

Vector constructions

2. *Construction of LCV1*

The lettuce chloroplast vector LCV1 consists of 4571 bp of lettuce chloroplast genome sequence with a unique 16 bp *Pac*1/*Asc*1 site added (**Figure 1**), cloned into *Sac*1/*Kpn*1 restriction sites on the polylinker of a pBluescript SK+ backbone vector (**Figure 2**). The lettuce sequence spans from the *rps*7/3'-*rps*12 intergenic region to the 16SrRNA/*trn*I intergenic region and corresponds to nucleotide positions 100021-104387 in the tobacco chloroplast genome (GI accession number Z00044). An alignment of this lettuce sequence with the tobacco chloroplast genome sequence is given in **Figure 3**. The following description of the construction of LCV1 is outlined in **Figure 4**.

Four primers LCV1A, LCV1B, LCV1C and LCV1D were used to amplify this region in two halves (LCV1A-B and LCV1C-D) and to introduce a unique *Pac*1/*Asc*1 restriction site in the ORF70B/*trn*V intergenic region at the position corresponding to nt 102367 in the tobacco chloroplast genome sequence. DNA from clone 6 of the *Sac*1 library of the lettuce chloroplast genome (Jansen and Palmer, Current Genetics 11: 553-564 (1987)) was used as a template for the LCV1 vector. LCV1A and LCV1B amplified a 2575 bp fragment (2551 bp lettuce sequence + 24 bp extension) LCV1A-B spanning from the *rps*7/3'-*rps*12

intergenic to the ORF70B/*trn*V intergenic region (corresponding to 100021-102367 in the tobacco chloroplast genome). Primer LCV1A contains a *Sac*1 site and LCV1B contains *Pac*1/*Asc*1 sites so that *Sac*1 and *Pac*1/*Asc*1 sites are incorporated at the 5′ and 3′ end, respectively, of the LCV1A-B fragment.

The LCV1 A-B fragment was cloned into the *E.coli* plasmid vector PCR2.1 to create PCR2.1 LCV1A-B. These clones were screened for orientation using *Sac*1 and *Sac*1+*Xba*1. The *Sac*1/*Xba*1 insert was cloned into the polylinker of pBluescript to create pBSLCV1 A-B.

Primers LCV1C and LCV1D amplified a 2042 bp fragment (2020 bp lettuce sequence + 22 bp extension) LCV1 C-D. The LCV1C primer contains *Pac*1/*Asc*1 sites and the LCV1D primer contains a *Kpn*1 site so that a *Pac*1/*Asc*1 and a *Kpn*1 site are added to the 5′ and 3′ end, respectively, of the LCV1 C-D fragment. The LCV1 C-D fragment was cloned into PCR2.1 to create PCR2.1 LCV1 C-D. For the final cloning step, PCR2.1 LCV1 C-D was restricted with *Asc*1+*Kpn*1 to release a 2031 base pair insert that was ligated to pBS A-B, which was linearised with *Asc*1+*Kpn*1, creating LCV1.

2. *Construction of LCV2*

LCV2 consists of a 2253 bp lettuce chloroplast genome sequence (**Figure 5**) spanning from the 16S rRNA/*trn*I intergenic region to the *trn*A/23S rRNA intergenic region, cloned into the PCR2.1 (Invitrogen) backbone vector (**Figure 6**). This sequence corresponds to nucleotide positions 104366-106260 in the tobacco chloroplast genome (GI accession number Z00044). An alignment of this lettuce sequence with the tobacco chloroplast genome sequence is given in **Figure 7**. The following description of the construction of LCV2 is outlined in **Figure 8**.

Four primers LCV2A, LCV2B, LCV2C and LCV2D were used to amplify this region in two halves (LCV2A-B and LCV2C-D) and to introduce unique *Pac*1/*Asc*1 restriction sites in the intergenic region between the *trn*I and *trn*A genes at the position corresponding to nucleotide 105370 in the tobacco chloroplast genome.

For the first half (A-B) of the vector, DNA from clone 6 of the *Sac*1 library of the lettuce chloroplast genome (Jansen and Palmer, Current Genetics 11: 553-564 (1987)) was used as a template. Primers LCV2A and LCV2B amplified a 1258 bp fragment (1242 bp lettuce sequence + 16 bp extension) (LCV2A-B) spanning from 16SrRNA/*trn*I intergenic region to the *trn*I/*trn*A intergenic region. This fragment was cloned into the *E. coli* plasmid cloning vector PCR2.1 (Invitrogen) to create PCR2.1 LCV2A-B. Primer LCV2B contains *Pac*1/*Asc*1 sites so that the LCV2A-B fragment has *Pac*1/*Asc*1 sites at the 3'end. PCR2.1 LCV2 A-B clones were screened for orientation by digestion with *Kpn*1/*Asc*1, which releases a fragment of approximately 1300 bp, and *Xba*1/*Asc*1 which linearised clones with the correct orientation for subsequent cloning.

For the second half of the vector chloroplast DNA from lettuce cultivar Evola (Leen de Moss seeds) was used as a template because the entire *trn*A gene was not contained in a single clone in the lettuce chloroplast genome library. Primers LCV2C and LCV2D amplified a 1011 bp fragment (995 bp lettuce sequence + 16 bp extension) LCV2C-D. This sequence spans from the *trn*I/*trn*A intergenic region to the *trn*A/23S rRNA intergenic region. Primer LCV2C contains *Pac*1/*Asc*1 sites so the fragment LCV2C-D has *Pac*1/*Asc*1 sites at its 5' end. This fragment was cloned into PCR2.1 to create PCR2.1 LCV2 C-D. These clones were screened for orientation using *Kpn*1+*Asc*1, which linearises clones with required orientation and *Xba*1+*Asc*1, which releases a fragment of approximately

1000 bp in clones with the required orientation. To generate LCV2, the 1.3 kb *Asc*1+*Xba*1 insert from PCR2.1 LCV2C-D was subcloned into PCR2.1 LCV2A-B linearised with *Asc*1+*Xba*1.

3. *Construction of LCV1-MSK18 and LCV2-MSK18*

MSK18 is an expression cassette adapted from pMSK18 (Hibberd *et al.*, The Plant Journal 16, 627-632 (1998)). Plasmid MSK18 was a gift from John Gray (Dept. Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK). Full details of the construction of pMSK18 have been described previously (Hibberd *et al.* 1998, supra). The MSK18 expression cassette consists of the mGFP coding region (Haselhoff *et al.*, Trends in Genetics 11, 328-329 (1997)) fused to a bacterial *trc* promoter (Amman and Brosius, Gene 40, 183-190 (1985)), and an *aad*A coding region, derived from pUC-atpX-AAD (Goldschmidt-Clermont, Nucleic Acids Research 19, 4083-4089 (1991)) fused to a tobacco *rrn* promoter derived from pZS197 (Svab and Maliga, Proc. Natl. Acad. Sci USA 90, 913-917 (1993)). A tobacco *psb*A 3′ UTR derived from pSZ197 (Svab and Maliga, 1993 supra) is fused to the 3′ end of the *aad*A gene (**Figure 9**).

Using pMSK18 as a template, *Pac*1 and *Asc*1 sites were added by PCR amplifying the cassette with primers containing *Pac*1 (5′) and *Asc*1 (3′) restriction sites to 5′ and 3′ ends of the of the MSK18 expression cassette. The primers used for this were MSK18 A (Forward) 5′-tagttaattaaTTGACAATTAATCATCCGGCTCGT-3′ (**SEQ ID NO:31**) and MSK18 B (Reverse) 5′-taggqcgcgccTCGAATATAGCTCTTCTTTCTTA-3′ (**SEQ ID NO:32**). The MSK18 A-B PCR product was cloned into PCR2.1 to create PCR2.1 MSK18. PCR2.1 MSK18 was restricted with *Pac*1/*Asc*1 to release the MSK18 insert that was cloned into the *Pac*1/*Asc*1 sites in LCV1 and LCV2 to create LCV1-MSK18 (**Figure 9**) and LCV2-MSK18 (**Figure 10**).

EXAMPLE 2

Construction of LCV2-LEC1

Lettuce expression cassette 1 (LEC1; **Figure 21**) contains the *aad*A gene, which confers spectinomycin and streptomycin resistance in plants, and the influenza virus haemagglutinin gene (HA) that codes for a potential influenza sub-unit vaccine. Both genes are under the control of a single lettuce specific promoter (P*rrn*) and terminator sequence (3' *psb*A). A chloroplast ribosome-binding site also precedes both genes. The expression cassette was assembled in three pieces using a combination of PCR amplification and overlap extension (**Figure 22**).

The lettuce chloroplast specific ribosomal RNA operon promoter (P*rrn*) was amplified from lettuce chloroplast DNA (SacI fragment 6 from the Jansen cpDNA library; Jansen and Palmer, (Current Genetics 11: 553-564 (1987)) using PCR primers A and B. The *aad*A gene and upstream ribosome-binding site (rbs) was amplified from the tobacco chloroplast transformation vector pZS197 using PCR primers C and D. The HA gene and upstream rbs was amplified from an in-house HA gene construct (HA con3) using PCR primers E and F. A lettuce specific *psb*A termination sequence (3'*psb*A) was amplified from lettuce chloroplast DNA (cv. Evola) using PCR primers G and H. PCR products A+B and C+D were fused by overlap extension using PCR primers A and D.

The resulting PCR product A+D was cloned into the Sac1/NotI sites of pBS SK+ to create pBS A+D. PCR product E+F was cloned into the NotI/BamHI sites of pBS SK+ to create pBS E+F. PCR product G+H was cloned into the BamHI/PstI sites of pBS E+F to create pBS E+H. The complete insert (E+H) was excised by restriction with NotI/PstI and cloned into the NotI/PstI sites on pBS A+D to create pBS SK+ LEC1.

Expression of *aadA* and HA in pBS SK+ LEC1 was tested in *E.coli*. Transformed *E.coli* cells were resistant to streptomycin indicating that the *aadA* gene was expressed. Western analysis of HA expression with anti-HA sera showed expression of HA in *E.coli*. The entire expression cassette (P*rrn*/*aadA*/HA/*psbA*) was excised from pBS SK+ LEC1 using the restriction enzymes PacI and AscI and cloned into the PacI/AscI sites on the lettuce chloroplast transformation vector LCV2 to create LCV2-LEC1.

EXAMPLE 3

Obtaining seedlings and an *in vitro* stock of plants

Protoplasts of plants are isolated from leaf material of donor plants. In this example the obtaining of leaf shoot cultures is given.

Seeds are sterilized by subsequent washing in 70% ethanol, 0.7% NaOCl solution during 20 minutes and three times washing with sterile demineralized water. Seeds are sown on Murashige and Skoog (Murashige and Skoog, Physiol. Plant., 15: 473-497 (1962)) medium with saccharose 2%, without hormones. Preferably, seeds can be cultured at 15°C for 2 days in the dark, after which the seeds are transferred to 25°C in the light (approximately 3000 lux, photo period 16 hr light/8 hr dark TL FTD 840). When first true leaves appear, shoot tips are transferred to Murashige and Skoog based medium with 3% saccharose, without hormones. These sterile shoot cultures are grown under similar growth conditions.

EXAMPLE 4

Isolation of protoplasts

Three week old shoot cultures are used for isolation of protoplasts. Leaves are cut into small pieces and preplasmolysed during 1 hr in the dark in PG solution (54.66 g/l sorbitol and 7.35 g/l $CaCL_2.2H_2O$). The PG solution is then replaced by an enzyme solution with 1% cellulase and 0.25% macerozym. Incubation takes place during 16 hrs in the dark at 25°C.

Subsequently, the suspension is filtered through a nylon mesh filter (41µm) en washed with a third of a volume of CPW16S solution (Frearson et al., Developmental Biology 33:130-137 (1973)) by centrifugation at 700 rpm during 8 minutes. In this way, intact protoplasts are collected on the surface of the supernatant. Protoplasts are washed in W5 solution (9 g/l NaCl, 18.38 g/l $CaCl_2.2H_2O$, 0.37 g/l KCl, 0.99 g/l glucose, 0.1 g/l Morpholinoethanesulfonide buffer (MES)) by centrifugation at 600 rpm during 5 minutes. With the procedure described, a protoplast yield of approximately 10-15 x 10^6 protoplasts per gram leaf material can be obtained.

EXAMPLE 5

Selection of protoplast derived calli on spectinomycin resistance

Protoplasts of lettuce, derived as described in example 4, are diluted in culture medium ½ B5 (Gamborg et al. Exp. Cell Res. 50:151 (1968)): 375 mg/l $CaCl_2.2H_2O$, 18.35 mg/l NaFeEDTA, 270 mg/l sodium succinate, 103 g/l saccharose, 0.1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D), 0.3 mg/l 6-benzylaminopurin (BAP) and 0.1 g/l MES and set to a culture density of 6 x 10^4 protoplasts per ml.

The protoplast suspension is mixed 1:1 with ½ B5 culture medium with agarose. The agarose beads are plated in

larger petri dishes with liquid ½ B5 culture medium on top of it.

The petri dishes are taped with parafilm and cultured at 25°C in the dark. One week after initiation of culture the culture medium is diluted with fresh liquid ½ B5 culture medium and 0.1 g/l MES. The cultures are transferred to the light (approx. 3000 lux, photo period 16 hours light/8 hours dark, TL FTD 840).

When calli are about 0.5 mm in size they are transferred to callus growth medium SH2 (Schenk & Hildebrandt, Can. J. Bot. 50:199-204 (1972)) with 30 g/l saccharose, 5 g/l agarose, 0.1 mg/l 1-naphtalene acetic acid (NAA) and 0.1 mg/l benzylaminopurin (BAP), and the selecting agent spectinomycin dihydrochloride at concentrations of 0-1000 mg/l. It was found that the optimal concentration of selection is 500 mg/l. The non-resistant calli appear as white calli. They also grow slower as compared to the control calli. The culture conditions are as described above for the above protoplast calli.

EXAMPLE 6

Transformation of protoplasts with polyethylene glycol and selection for *aad*A encoded antibiotic resistance

Protoplasts of lettuce, derived as described in example 4, are set to a density of approximately 1-1.5 x 10^6 protoplasts/0.4-0.6 ml in transformation buffer (0.4 M mannitol, 15 mM $MgCl_2$, 1% (w/v) MES, pH 5.8). Subsequently, 10 µl of plasmid suspension (1 µg DNA/µl sterile H_2O) is added to the protoplasts as well as 0.4-0.6 ml PEG solution (40% w/v PEG 6000, 2.36 g/l $Ca(NO_3)_2.4H_2O$ and 7.28 g/100ml mannitol). Incubation is performed at room temperature for 5-30 minutes. Protoplasts are washed and resuspended in culture medium $^1/_2$ B5 (Gamborg et al., Exp. Cell Res. 50:151 (1968)): 375 mg/l $CaCl_2.2H_2O$, 18.35 mg/l NaFeEDTA, 270 mg/l sodium

succinate, 103 g/l saccharose, 0.1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D) and 0.3 mg/l 6-benzyl aminopurin (BAP).

The protoplast suspension is mixed 1:1 with ½ B5 culture medium with agarose. The agarose beads are plated in larger petri dishes with liquid ½ B5 culture medium on top of it.

The petri dishes are taped with parafilm and cultured at 25°C. After 6 days selection of the microcalli is performed by adding 500 mg/l of the selective agent spectinomycin dihydrochloride (final concentration). One week after initiation of culture the culture medium is diluted with fresh liquid ½ B5 culture medium, with addition of spectinomycin dihydrochloride and cultured in the light (approx. 3000 lux, photo period 16 hours light/8 hours dark, TL FTD 840).

When calli are about 0.5 mm in size they are transferred to callus growth medium SH2 (Schenk & Hildebrandt, 1972, supra) with 30 g/l saccharose, 5 g/l agarose, 0.1 mg/l 1-naphtalene acetic acid (NAA) and 0.1 mg/l benzylaminopurin (BAP), and the selecting agent spectinomycin dihydrochloride at concentrations described above. Culture conditions are as described above.

After 2 weeks calli are transferred to regeneration medium SHreg (Schenk and Hildebrandt, 1972, supra) with 15 g/l saccharose, 15 g/l maltose, 0.1 mg/l NAA and 0.1 mg/l BAP and spectinomycin dihydrochloride in concentrations described above. Spectinomycin resistant calli appear as green calli amongst white (non-resistant) calli.

Regenerating plants appear after approximately 6 weeks and furtheron, and are transferred to rooting medium (Schenk and Hildebrandt, supra) with 30 g/l saccharose and 8 g/l agar with the concentrations of spectinomycin dihydrochloride mentioned above. Alternatively, in

transformation vectors where gfp (green fluorescent protein) is added as the gene of interest, gfp fluorescence is detected using an inverted microscope with the proper filter combinations. Green calli were detected 4-5 weeks after initiation of each experiment.

Table 1 gives an overview of the results obtained in protoplast transformation experiments with three different plasmids. Spectinomycin resistant calli were obtained after transformation of protoplasts with the plasmids PLCV2-MSK18 and PLCV2-LECI. Approximately 40-50% of the protoplasts did survive the PEG treatment. Callus lines of each individual event are maintained on medium SHreg with the selective agent spectinomycin dihydrochloride and yielded regenerated plants from plasmids pLCV2-MSK18 and pLCV2-LEC1 (**Table 1**). Also, ploidy differences were observed between individual calli.

Table 1. Selection of plastid transformants

Treatment/ Experiment	# pps treated	# green calli	# regenerating calli
control	none	0	
control + PEG	1,26 x 10^6	0	
pLCV1-MSK18	1,26 x 10^6	0	
pLCV2-MSK18/exp 1	1,26 x 10^6	1	0
pLCV2-MSK18/exp 2	2,40 x 10^6	1	0
pLCV2-MSK18/exp 3	4,80 x 10^6	5	2 (1 ++, 1+/-)
pLCV2-LEC1/exp 1	3,60 x 10^6	5	3 (1 ++, 2 +/-)

The transgenic callus has been obtained using vectors with specific lettuce chloroplast DNA homologous sequences. Selection of transformed cells with the non-lethal selective agent spectinomycin has been successful. The optimal transformation frequency for lettuce, determined as the

number of green calli to the number of surviving protoplasts is about 1 in 3-6.10^5 protoplasts (Table 1).

The plants obtained from transformation experiments with pLCV2-LEC1 were found to have a normal, diploid ploidy level and showed a normal growth. Seed-set after selfing was obtained from these plants (**Figure 11**).

EXAMPLE 7

Transformation of protoplasts via electroporation and selection on *aadA* encoded antibiotic resistance

Protoplasts, derived as described in example 4, are suspended in transformation buffer HBS (150 mM KCl, 4 mM $CaCl_2.2H_2O$, 10 mM HEPES (pH 7.2)), and enough mannitol to osmotically balance the protoplasts. This is dependent on the genotype but it can easily be found out experimentally. Aliquots of 1 x 10^6 protoplasts/0.5 ml HBS buffer and mannitol are put into a conical centrifuge tube, and plasmid DNA solution is added. Plasmid DNA concentrations in the transformation buffer should preferably be in the range of 10-100 µg/ml. The protoplast-DNA suspension is transferred to the electroporation chamber and electroporated using a single electric pulse (e.g. 325µF, 300 V) The optimal setting can vary with species and cell type, and should be determined in preliminary experiments. The most efficient parameters are set by finding the pulse settings that result in 50% protoplast death by 24h after the shocks. More details of the method are described by G.W. Bates (Plant transformation via protoplast electroporation. From: Methods in Molecular Biology Vol 111: Plant cell Culture Protocols, Pp 359-366 (1999)).

After electroporation, protoplasts culture and selection is performed as described in example 6.

EXAMPLE 8

Adjustment of spectinomycin threshold levels in cotyledons

For the adjustment of the optimal concentration of spectinomycin, for selection of cells with chloroplasts/ plastids, which are transformed with constructs having the *aad*A gene as selectable marker, 4-10 day old cotyledons were plated with the abaxial side onto MS medium (Murashige and Skoog, supra) with 0.8% agar, 30 g/l saccharose, 100-200 mg/l carbenicillin, 0.1 mg/l benzylaminopurin (BAP), 0.1 mg/l 1-naphtalene acetic acid (1-NAA) at pH 5.8, and with various concentrations of spectinomycin dihydrochloride. The cotyledons were obtained as described in Example 3, and cultured at 25°C in the light (approx. 3000 lux, photo period 16 hours light/8 hours dark, TL FTD 840). It was found that a concentration of 0.5-1 g/l spectinomycin dihydrochloride was sufficient for efficient selection, leading to complete bleaching and loss of growth and regeneration of control cotyledons.

EXAMPLE 9

Transformation of plant material via biolistics and selection for *aadA* encoded antibiotic resistance

For bombardment of cotyledons, seeds were sown as described in example 3. Alternatively, leaf pieces can be used as explant material for shooting, under similar conditions. Cotyledons (3 to 12 days old) or leaf pieces from 10-14 days old seedlings are placed with the abaxial side onto MS medium (Murashige and Skoog, supra) with 0.8% agar, 0.3 mg/l BAP and 0.1 mg/l 2,4-D (pH 5.8) and preincubated for 1-6 days before transformation with a particle gun.

The cotyledons are cultured at 25°C in the light (approx. 3000 lux, photo period 16 hours light/8 hours dark, TL FTD 840).

Gold particles (0.6 to 1.6 µm) were prepared for transformation by mixing 50 µl of suspension (60 mg/ml 50% glycerol) with 5 µg DNA (1µg/µl H_2O), 50 µl $CaCl_2$ (2.5 M) and 20 µl spermidine (0.1 M base). The particle-DNA mixture was incubated at room temperature for 1-3 minutes and centrifuged for 3-10 sec. in an Eppendorf centrifuge. After removal of the supernatant, the coated particles are washed and diluted in 48-60 µl ethanol. The particles (6-8 µl per carrier) are applied to the macrocarrier holders and the bombardment is performed with PDS-1000/He Biolistic particle delivery system (BioRad).

The explants are placed at approximately 6 cm target distance and bombarded using a 1100 p.s.i rupture disc. Details of the procedure has been described by Klein et al. (Bio/Technology 6: 559-563 (1988)).

Two to fourteen days after bombardment, the cotyledons are transferred to MS1 liquid medium (Murashige and Skoog, supra) with 30 g/l saccharose and supplemented with 100-200 mg/l carbenicillin, 0.1 mg/l benzylaminopurin (BAP) and 0.1 mg/l 1-naphtalene acetic acid (1-NAA) at pH 5.8 as described above with the addition of a selective agent (e.g. spectinomycin dihydrochloride at concentration of 500 mg/l). They are incubated in liquid medium at 25°C in the light (approx. 3000 lux, photo period 16 hours light/8 hours dark, TL FTD 840) for about 1-8 days, after which they are transferred to solid MS1 medium (see above with the addition of 8 g/l agar). Cultures are transferred onto fresh medium every 2 weeks.

When green callus or shoots appear, they are transferred to medium MS1 without carbenicillin, but including the selective agent spectinomycin dihydrochloride.

Table 2 presents results from transformation experiments with pLCV2-MSK18. It was found that green,

spectinomycin resistant callus was formed on bombarded cotyledons, approximately 2.5 months after initiation of the experiment. The spectinomycin resistant callus was maintained on MS1 medium with the selective agent.

Table 2. Results of particle bombardment experiments with pLCV2-MSK18 using cotyledons or leaf pieces.

Explant type/treatment	Number of bombarded explants	Number of explants with spectinomycin resistant callus
Cotyledon, bombarded selection	180	1
Cotyledon control selection	30	0
Leaf bombarded selection	96	0
Leaf control selection	16	0

EXAMPLE 10

Molecular analysis of spectinomycin resistant calli of lettuce

Spectinomycin resistance of plant cells may be the result, apart from transformation with the vector LCV2-MSK18, of spontaneous mutation of chloroplast DNA or insertion of the DNA into the nuclear genome. Therefore, the callus and regenerated plants were screened for the integration of the right and left homologous border segment as is described in this Example. Additionally, it was determined whether the *aadA* gene, the gfp and HA gene were correctly integrated in the chloroplast DNA.

1. *Analysis of calli derived from PEG protoplast transformations with pLCV2-MSK18*

Spectinomycin resistant callus of lettuce was analysed by PCR using different primer combinations to confirm the integration of the plasmid pLCV2-MSK18 in the genome of the chloroplast.

As an endogenous control for chloroplast DNA amplification, PCR analysis of the ATPase gene (Accession: AF162208) was carried out using the forward primer 5'-ACTAATAGTGGACAAATTGGC-3' (**SEQ ID NO:33**) and the reverse primer 5'-TTGCTTGATTGTATTTACTCG-3' (**SEQ ID NO:34**). To detect the presence of the selectable marker gene *Aad*A, the following primer combination was used: forward 5'-AAGTCACCATTGTTGTGCACG-3' (**SEQ ID NO:35**) and reverse 5'-TATGACGGGCTGATACTGGGC-3' (**SEQ ID NO:36**). In order to demonstrate the physical integration of the plasmid into the chloroplast genome 2 primer combinations were developed which amplify hybrid regions of the plasmid and the chloroplast genome (see **Figure 12**). The first primer combination consisting of P1 and P2 amplifies the junction containing the *trn*I sequence of the chloroplast genome (left border integration). The second primer combination consisting of P3 and P4 amplifies the junction containing the *trn*A sequence of the chloroplast genome (right border integration).

Total DNA was isolated from spectinomycin resistant callus using a commercially available DNA isolation kit from Sigma (Genelute Plant Genome DNA Kit). The PCR reaction was carried out using a total amount of 30 ng DNA after which the reaction products were analysed on a 1% agarose gel.

The result of the analysis of 5 independent spectinomycin resistant calli derived from PEG protoplast transformations is shown in **Figure 13** (data of 2 calli not shown but identical to the other 5). The ATPase fragment of

about 424 bp is only present in callus material and leaf material of lettuce, and as expected not visible for the pLCV MSK18 DNA (**Figure 13A**). PCR amplification of the *aad*A gene gave the expected fragment of approximately 413 bp for the transgenic callus and the plasmid pLCV2-MSK18 (**Figure 13B**).

To confirm the integration of the pLCV2-MSK18 vector into the lettuce chloroplast genome, the two primer combinations were used which specifically detect either one of the two junctions which emerge after integration of the plasmid by homologous recombination. The integration on *trn*I junction was investigated using the PCR primers indicated above, which resulted in an expected band of approximately 2000 bp as well (**Figure 13C**). **Figure 13D** shows the amplification of the *trn*A junction which results in an expected band of approx. 1500 bp in the spectinomycin resistant callus. The results of this analysis confirm the transplastomic nature of the obtained spectinomycin resistant pLCV2-MSK18 lettuce calli, and no escapes were found.

For further confirmation of integration, the left and right integration junctions were amplified by PCR using primer pairs P1+P2 and P3+P4. The PCR products from one spectinomycin resistant callus sample were cloned into PCR2.1 and sequenced using M13 forward and M13 reverse primers. These sequences confirmed that LCV2-MSK18 was integrated in the lettuce chloroplast genome (**Figure 14**).

To eliminate the possibility of amplification of unintegrated LCV2-MSK18 plasmid DNA, primers P1 and P4 were designed from lettuce chloroplast sequences external to the vector target region (**Figure 12**). PCR analysis was carried out on DNA isolated from 6 putatively transformed calli. In all cases, P1 and P4 give two PCR products, a 2476 bp band corresponding to the expected size of a product amplified from an untransformed wild-type chloroplast genome, and a 4623 bp band corresponding to the size of a PCR product

expected from a transformed chloroplast genome. **Figure 15** shows the results in detail for one callus, and **Figure 16** shows the PCR results on insert integration for 6 independent calli.

2. *Molecular analysis of spectinomycin resistant callus, derived after biolistic transformation with pLCV2-MSK18*

Similar primer combinations, as used for the spectinomycin resistant callus out of PEG protoplast experiments were used to evaluate the transplastomic nature of the callus derived from bombarded tissue. **Figure 17** shows the products of the *trn*I and *trn*A junction, respectively. It was verified that the callus was of a transplastomic nature.

3. *Molecular analysis of putative transplastomic callus, derived from PEG protoplast transformation experiments with pLCV2-LEC1.*

For the analysis of the calli, obtained by protoplast transformation experiments with pLCV2-LEC1, similar primer combinations as for the pLCV2-MSK18 plasmid transformations could be used for the aadA gene, the endogenous control and the insert integration P1 + P4 (**See Figure 13**). Furthermore, PCR analysis on left border integration was performed by using the forward primer 5'-ACTGGAAGGTGCGGCTGGAT-3' (**SEQ ID NO:37**) and the reverse primer 5'TATGACGGGCTGATACTGGGC-3' (**SEQ ID NO:38**). Right border integration was performed by using the forward primer 5'-ATGCAAAAACTTCCCGGAAAT-3' (**SEQ ID NO:39**) and reverse primer 5'-CTCGCCCTTAATTTTAAGGC-3' (**SEQ ID NO:40**).

Results of these analyses are shown in **Figure 18**. It is clear that all 5 independent calli are true transplastomic ones, and no escapes were found.

4. Molecular analysis of regenerated plants from transplastomic callus, derived from PEG protoplast transformation experiments with pLCV2-MSK18 and LEC1

Figure 19 shows the PCR results from DNA derived from several plants regenerated from one transplastomic pLCV2-MSK18 callus. **Figure 20** shows PCR analysis of pLCV2-LEC1 regenerated plants. It is clear that both types of plants are truly transplastomic.